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^{1}H-NMR study of the λ operator site O_L1: assignment of the imino and adenine H2 resonances

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ABSTRACT

One- and two-dimensional proton NMR methods are being used to study the synthetic λ operator site O_L1, a 17 base-pair DNA duplex recognized by λ repressor and Cro protein. The complete assignment of the 17 imino protons, which participate in Watson-Crick hydrogen bonding, and of the eight adenine H2 protons, which lie in the minor groove of the double helix, is presented.

INTRODUCTION

The recent determination of the three-dimensional structures of Cro and the operator-binding domain of λ repressor has focused attention on the molecular basis of protein-DNA recognition (1-4). These proteins regulate gene expression by binding to six specific operator sites in the phage λ genome. These sites are 17 base pairs in length, have similar sequences and are almost symmetric about the central base pair (5). The ability of Cro and λ repressor to discriminate among these sites is essential to the development of λ phage. The specificity of these protein-DNA interactions and their modulation from site to site pose a challenging problem in structural biology. We have begun a one- and two-dimensional proton NMR study of a 17 base-pair synthetic oligonucleotide whose sequence is that of O_L1, the strongest λ repressor binding site in the phage genome. We report here the complete assignment of the imino protons, which participate in Watson-Crick hydrogen bonding, and of the adenine H2 protons, which lie in the minor groove of the double helix. Because these resonances are sensitive to DNA conformation, dynamics and interactions, we anticipate that their assignment will be useful for the analysis of the interaction of O_L1 with Cro and λ repressor.

MATERIALS AND METHODS

The O_L1 oligonucleotide was obtained from PL Biochemicals and provided to us by C.O. Pabo. The single strands were annealed as described (6) and exhaustively dialyzed against 10 μM EDTA. After dialysis the solution

was lyophilized; the powder was dissolved in either buffer A or buffer B (see below) to a final duplex concentration of 60 mg/ml, assuming an optical density at 260 nm of 21 cm^{-1} for a 1 mg/ml solution. NMR experiments were performed at 498 Megahertz. Water elimination in H_2O solution was achieved by the Redfield long-pulse technique (7). One-dimensional Nuclear Overhauser Effect (NOE) difference spectra were measured with a presaturation time of one second (8). The irradiated resonance was only half-saturated to minimize non-selective and second-order effects. Spectra in D_2O solution were obtained in buffer A only. Two-dimensional Nuclear Overhauser Effect spectra (NOESY) were obtained by a modification of the pure-phase method of States et al (9). Two mixing times -- the first 100-350 ms, the second 5 ms -- were interweaved and, after appropriate scaling, subtracted for each t_1 value. This 2D difference method eliminates echoes and artifacts, and suppresses the diagonal peaks. 4096 points were sampled over a 4500 hz sweep width in t_2; after Fourier transformation on the fly, 1024 points in the aromatic region were extracted and stored. A mild convolution difference with parameters 2,100,.9 was applied. 261 t_1 values were obtained and zero-filled to 1024.

Buffer A consists of 200 mM KCl, 50 mM potassium phosphate (pD 7.4), 1 mM sodium azide and 1 mM EDTA. Buffer B consists of 10 mM Tris-HCl (pH 7.4 at room temperature), 1 mM sodium azide and 1 mM EDTA. D_2O buffers contain 99.96% D_2O; H_2O buffers contain 15% D_2O.

RESULTS

O_L1 is an asymmetric duplex with sequence

$$5' \; T_1A_2C_3C_4A_5C_6T_7G_8G_9C_{10}G_{11}G_{12}T_{13}G_{14}A_{15}T_{16}A_{17} \; 3'$$
$$3' \; A_1T_2G_3G_4T_5G_6A_7C_8C_9G_{10}C_{11}C_{12}A_{13}C_{14}T_{15}A_{16}T_{17} \; 5'$$

In the numbering scheme shown, T_1 is the first (5') base of the upper strand, A_1 the last (3') base of the lower strand, etc. We discuss first the assignment of the imino resonances by the one-dimensional NOE method of Redfield (7,8,10-15). We then describe how a combination of one- and two-dimensional methods were used to assign the adenine H2 resonances.

Imino Assignments

O_L1 contains eight AT and nine GC base pairs. The corresponding imino resonances are shown in panel A of Figure 1. At 10°C there are six resolved AT resonances downfield [labelled α_1 - α_{78} in Figure 1] and eight resolved GC resonances upfield [labelled γ_1 - γ_9]. As the temperature is increased, these

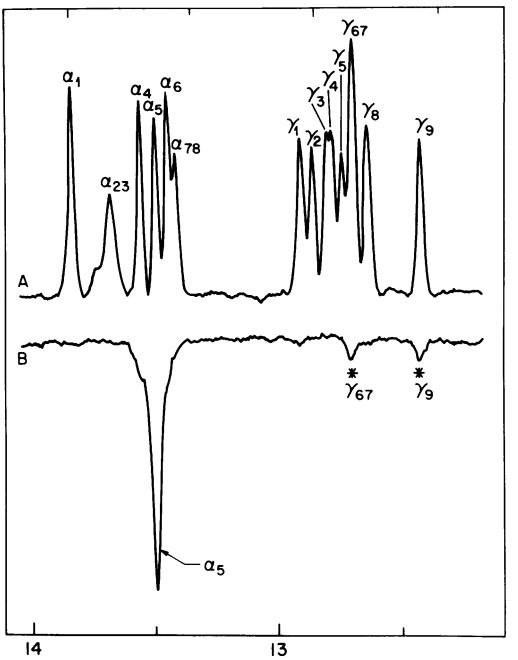

Figure 1. (A) Imino resonances at 10°C of O.1. Peaks α_1-α_{78} arise from AT base pairs; γ_1-γ_9 from GC base pairs. The spectrum is the average of 1480 transients. An exponential convolution difference with parameters 1 Hertz, 20 Hertz, and subtraction ratio 0.95 was applied with polynomial baseline correction. (We define these parameters such that the final spectrum is the difference between (i) a spectrum which has undergone Lorentzian line-broadening of 1 Hertz; and 0.95 times (ii) a spectrum which has undergone Lorentzian line-broadening of 20 Hertz.) (B) Interbase-pair Nuclear Overhauser Effect from AT imino resonance α_5 to neighboring imino protons γ_{67} and γ_9. This data corresponds to row 4 of Table 1. The preirridation time was one second. The irradiated resonance was only half-saturated to minimize non-selective and second-order effects. An exponential linebroadening of 2 Hertz was applied. The sodium deoxyribonucleate was made 6 mM in buffer B in 85% H_2O solution.

resonances are observed to broaden sequentially and lose intensity (data not shown). This is due to base-pair fraying with consequent solvent exchange. Such fraying usually proceeds from the ends inward as the temperature is raised (14,15). Resonances α_{23} and α_{78} each represent two protons which have already lost most of their intensity at 10°C by this mechanism. Since α_{23} loses intensity at the lowest temperature, it is assigned as the resonance arising from the terminal AT base pairs 1 and 17. Since α_{78} is next to broaden, it is assigned to the penultimate base pairs 2 and 16.

Table 1. Summary of interbase-pair Nuclear Overhauser Effects. As described in the text, α represents an AT imino resonance, γ a GC imino resonance, and X an interbase-pair NOE. Each row represents a 1D NOE difference spectrum. Selective irradiation of resonance α_1, in row 1 for example, leads to NOEs observed at γ_4 and γ_8. (X) denotes effects which may in part be non-selective. This is a technical problem in crowded spectral regions.

	α_1	α_{23}	α_4	α_5	α_6	α_{78}	γ_1	γ_2	γ_3	γ_4	γ_5	γ_{67}	γ_8	γ_9
α_1	.	.	.	.	.	.	.	.	.	X	.	.	X	.
α_{23}	.	.	.	.	.	.	.	.	.	.	.	.	.	.
α_4	.	.	.	.	.	.	.	.	.	.	X	.	X	.
α_5	.	.	.	.	.	.	.	.	.	.	.	X	.	X
α_6	.	.	.	.	.	.	.	.	.	.	.	.	.	X
α_{78}	.	.	.	.	.	.	.	.	.	.	.	.	.	.
γ_1	.	.	.	.	.	.	.	(X)	.	.	.	X	.	.
γ_2	.	.	.	.	.	.	(X)	.	(X)	.	.	.	.	.
γ_3	.	.	.	.	.	.	.	(X)	.	.	.	.	.	.
γ_4	X	.	.	.	.	.	.	.	.	.	.	.	.	.
γ_5	.	.	X	.	.	.	.	.	.	.	.	.	.	.
γ_{67}	.	.	.	X	.	.	X	.	.	.	.	.	.	.
γ_8	X	.	X	.	.	.	.	.	.	.	.	.	.	.
γ_9	.	.	.	X	X	.	.	.	.	.	.	.	.	.

Figure 1(B) shows interbase-pair Nuclear Overhauser Effects (NOE's) from AT imino resonance α_5 to GC resonances γ_{67} and γ_9. Since NOE's identify neighboring spins in space, we may order these resonances in the duplex as 5'-$\gamma_{67}\alpha_5\gamma_9$-3' or 5'-$\gamma_9\alpha_5\gamma_{67}$-3'. In principle a complete list of such nearest neighbor relationships, together with one independent assignment, would uniquely assign the set of imino resonances even in the absence of a known sequence. In practice, this has not been obtained for $\underline{O_L}1$ because of resolution limitations and the absence of NOE's from the fraying terminal base pairs. However, because the $\underline{O_L}1$ sequence is known, a partial catalogue of nearest neighbor relationships is sufficient to permit a unique solution of the assignment problem.

Table 1 summarizes the NOE's observed between nearest-neighbor base pairs. No NOE's are seen from the terminal AT base pairs 1 and 17 (α_{23}) nor from the penultimate AT base pairs 2 and 16 (α_{78}). In addition, some GC resonances show only one nearest-neighbor NOE and not the expected two. This can be caused by the fraying of one of its neighbors (as would be the case for

G_3C_3) or by the fact that the resonance of its second nearest neighbor is too close in the spectrum to permit selective Overhauser effects to be observed. If both nearest neighbors have similar chemical shifts, selective NOE's are difficult to observe. This is a technical problem in crowded spectral regions.

We begin the analysis of Table 1 by rewriting the DNA sequence in terms of (AT) or (GC) base pairs. Let α represent an AT base pair, and γ a GC. The sequence of $\underline{O}_L$1 is then

$$\begin{array}{l} \quad\;\; 1 \qquad\qquad\qquad\qquad\quad 17 \\ 5'\ \alpha\alpha\gamma\gamma\alpha\gamma\alpha\gamma\gamma\gamma\gamma\gamma\alpha\gamma\alpha\alpha\alpha\ 3' \end{array}$$

We see that the sequence $\gamma\alpha\gamma\alpha\gamma$ occurs uniquely from base pairs 4-8. From Table 1 we observe that $\gamma_5\alpha_4\gamma_8\alpha_1\gamma_4$ form such a string of nearest neighbors. The proper 5'-3' polarity of this string is ambiguous, since $\gamma\alpha\gamma\alpha\gamma$ is symmetric. This ambiguity may be resolved on the basis of the relative temperature dependence of base-pair fraying. At 30°C only three AT resonances are observed: α_1, α_4 and α_5. Between 30°C and 50°C α_4 and α_5 broaden in concert whereas α_1 broadens only at higher temperatures. With the assignment of α_5 (given below) as AT base pair 13, this temperature data implies that the correct orientation is 5-$\gamma_5\alpha_4\gamma_8\alpha_1\gamma_4$-3'. With this choice of polarity α_4 and α_5 are assigned to the symmetry-related sequences 5'-CA_5C-3' and 5'-CA_{13}C-3' respectively. Their identical temperature dependence thus reflects their symmetric environments in the oligonucleotide. Resonance α_1 is assigned to A_7T_7; since this is the innermost AT base pair in the sequence, it is reasonable that it is the last to fray. We next observe that base pairs 12-15 form the string $\gamma\alpha\gamma\alpha$. Since base pairs 4-8 are already assigned, this string is unique among the remaining bases. From Table 1 we find that $\gamma_{67}\alpha_5\gamma_9\alpha_6$ forms such a string of nearest neighbors. There is no ambiguity in polarity for this asymmetric string, and so we now have the assignments

$$\begin{array}{l} \quad\;\; 1 \qquad\qquad\qquad\qquad\qquad\qquad 17 \\ 5'\text{-}\alpha\alpha\gamma\gamma_5\alpha_4\gamma_8\alpha_1\gamma_4\gamma\gamma\gamma\gamma_{67}\alpha_5\gamma_9\alpha_6\alpha\alpha\text{-}3' \end{array}$$

The GC resonance that frays at the lowest temperature is one of the two protons at γ_{67}. Since we have just assigned one of these protons to interior base pair 12, we may account for the second by assigning it to base pair 3. Together with our previous assignments of the terminal and penultimate AT base pairs, this gives

$$\overset{1}{5'-}\alpha_{23}\alpha_{78}\gamma_{67}\gamma_5\alpha_4\gamma_8\alpha_1\gamma_4\gamma\gamma\gamma\gamma_{67}\alpha_5\gamma_9\alpha_6\alpha_{78}\alpha_{23}\overset{17}{-3'}$$

We now find from Table 1 that γ_1 is next to γ_{67}, assigning the former to base pair 11. This leaves base pairs 9 and 10 to be assigned in some order to resonances γ_2 and γ_3. Although the effect from γ_1 to γ_2 may in part be non-selective, no NOE is observed between γ_1 and γ_3. On this basis we may order the resonances $\gamma_3\gamma_2\gamma_1$ to obtain the complete set of assignments:

$$\overset{1}{5'-}\alpha_{23}\alpha_{78}\gamma_{67}\gamma_5\alpha_4\gamma_8\alpha_1\gamma_4\gamma_3\gamma_2\gamma_1\gamma_{67}\alpha_5\gamma_9\alpha_6\alpha_{78}\alpha_{23}\overset{17}{-3'}$$

Thus a unique solution is obtained on the basis of partial nearest-neighbor NOE data, the sequential fraying of the duplex, and the DNA sequence.

Adenine H2 Assignments

The adenine H2 protons in the minor groove of B-DNA give rise to singlet resonances in the aromatic region of the proton NMR spectrum. This region also contains purine H8 and pyrimidine H6 resonances and is shown in Figure 2(A). An inversion-recovery pulse sequence may be used to identify the H2 protons selectively, as shown in Figure 2(B). This pulse sequence distinguishes between spins on the basis of their spin-lattice relaxation. Because H2 protons (in D_2O solution) have few nearby spins with which to interact, their relaxation is much slower than that of major groove protons. All eight adenine H2 resonances are resolved at 30°C. Thus, the terminal and penultimate pairs of H2 resonances have different chemical shifts despite the degeneracy of their respective imino resonances, α_{23} and α_{78} (see above).

Assignment of an AT imino proton ordinarily permits the immediate assignment of its H2 proton by means of their mutual cross-saturation (12,14,15). Specific irradiation of AT imino resonance α_6 (T_{15}), for example, gives rise to an NOE in the aromatic region, assigning the latter to A_{15}. This is shown in Figure 3. In this way the H2 resonances of A_5, A_7, A_{13} and A_{15} were assigned. However, this method has two limitations. First, if two imino resonances are not resolved, then their respective H2 resonances cannot be distinguished directly. Second, assignment of H2 resonances from base pairs near the ends of the duplex is restricted to the low temperatures at which their imino resonances are observable. At such low temperatures the H2 resonances are not always resolved.

Assignment of the terminal and penultimate H2 resonances in $\underline{O_L}1$ encounters both limitations, since the imino resonances α_{23} and α_{78} are not

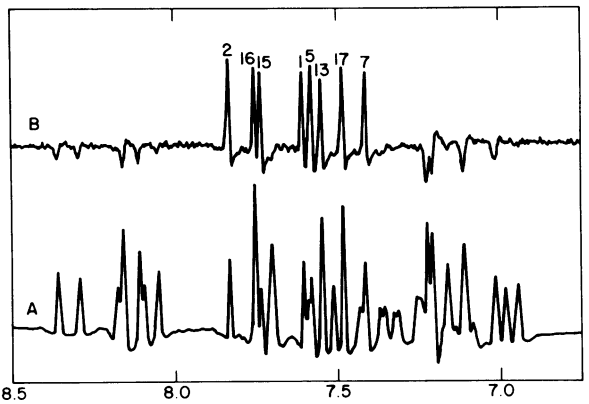

Figure 2. (A) The complete aromatic spectrum at 30°C of O_L1, which includes purine H8 and pyrimidine H6 resonances in addition to the adenine H2 resonances. The spectrum is the sum of 1182 transients with a recycle delay of 2 seconds. An exponential convolution difference with parameters 1 Hertz, 10 Hertz, and subtraction ratio 0.9 was applied before Fourier transformation. The oligonucleotide was made 6 mM in Buffer A in D_2O solution. (B) Inversion-recovery spectrum identifies the eight adenine H2 resonances at 30°C. A 1.2 second tau value was used in the 180-τ-90 pulse sequence with a recycle delay of two seconds. The spectrum is a sum of 164 transients. An exponential convolution difference with parameters 1 Hertz, 10 Hertz, and subtraction ratio 0.9 was applied. (We define these parameters such that the final spectrum is the difference between (i) a spectrum which has undergone Lorentzian line-broadening of 1 Hertz; and 0.9 times (ii) a spectrum which has undergone Lorentzian line-broadening of 10 Hertz.)

resolved and fray at the lowest temperatures. However, two-dimensional nuclear Overhauser effect spectra allow the individual assignment of these four H2 resonances.

We consider first the cross-relaxation that is possible among the adenine H2 protons. In principle the H2 protons found in contiguous AT sequences form a ladder of spins in the minor groove that could be followed by NOE's. The geometry of B-DNA suggests that only for 5'-TA steps will the successive H2 protons be sufficiently close to permit large NOE's to be observed (16). Moreover, local distortions of B-DNA seen in the crystal state (17-20) and in solution (21) shorten this distance (by compressing the minor groove). Similar local distortions lengthen the corresponding distance in 5'-AT steps (by compressing the major groove). The only contiguous AT base pairs in O_L1 occur at the ends, 5'-$T_1A_2C_3$ and 5'$T_{17}A_{16}T_{15}$. Thus 5'-T_1A_2 and 5'-$T_{17}A_{16}$ are the only 5'-TA steps in the operator, and only two strong inter-H2 NOE's should be observed.

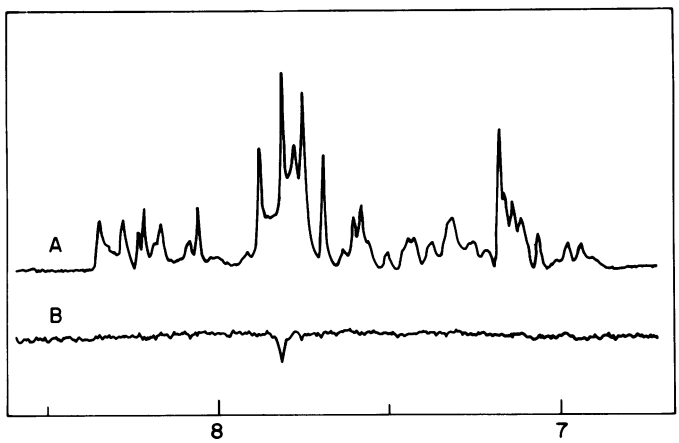

Figure 3. (A) Aromatic and amino resonances at 20°C of O_L1. The oligonucleotide was made 2.5 mM in Buffer A in 85% H_2O solution. The water resonance was suppressed by the Redfield long-pulse technique with a recycle delay of 0.1 sec. The differences between this spectrum and that shown in figure 2A are due to long-pulse distortions, incomplete relaxation between pulses, the presence of amino resonances, and small temperature effects. An exponential convolution difference with parameters 1,10,0.9 was applied. (B) Nuclear Overhauser Effect observed in the aromatic region following selective saturation of imino resonance α_6 ($A_{15}T_{15}$). The adenine H2 resonance of this base pair is immediately assigned. The presaturation time was one second. An exponential line broadening of 2 Hertz was applied.

Figure 4 shows a region of the NOESY spectrum at 0°C containing cross-peaks among the aromatic resonances. Only two cross-peaks are observed, corresponding to cross-relaxation between A_1H2 and A_2H2, and between $A_{17}H2$ and $A_{16}H2$. This indicates which terminal H2 is next to which penultimate H2, but does not distinguish between the two ends of the operator. We may continue by considering the spatial relationships between an H2 and its neighboring H1' protons in the minor groove. In standard B-DNA the nearest H1' protons are 4.5-5 A away: that of its own sugar, of its 3'-flanking sugar, and of its 5'-complementary sugar. These distances are expected to be sensitive to distortions in local structural parameters (17-20). In particular, the observed distortions in crystal structures can shorten by as much as one Angstrom the distance between an H2 and its 3'-neighboring H1'. Since the H1' assignments have been obtained by a two-dimensional method (6), H2-H1' relationships in principle enable the assignment of the H2 resonances. Figure 5 shows a region of the NOESY spectrum at 30°C containing cross-peaks between aromatic and H1' deoxyribose protons. From each penultimate H2 three effects are observed, the strongest between the H2 and the H1' of it 3'-neighbor. One

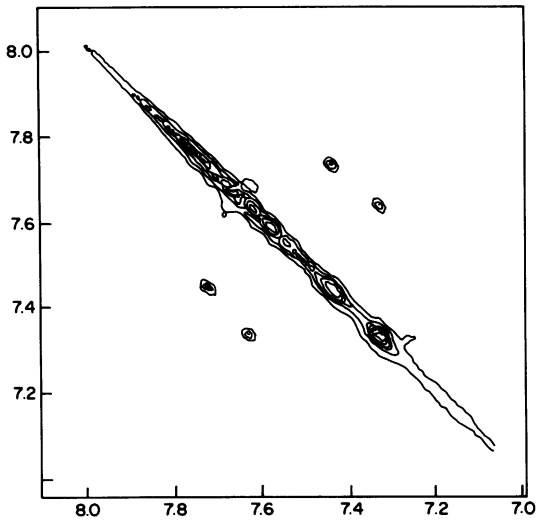

Figure 4. AH2-AH2 Nuclear Overhauser Effects are observed in the aromatic region of the pure-phase 2D NOESY spectrum of $\underline{O_L}1$ at 0°C. The oligonucleotide was made 6 mM in Buffer A in D_2O solution. A mixing time of 200 ms was used with a recycle delay of 2 seconds. A Gaussian line broadening of 3 hertz was applied in both dimensions.

penultimate H2 is observed to cross-relax with the H1' proton of C_3, whereas the other penultimate H2 is observed to cross-relax with the H1' proton of T_{15}. Thus the two ends of the operator are distinguished, and the assignment of the adenine H2 resonances is completed. Table 2 lists the complete set of H2 and imino assignments.

DISCUSSION

The determination of the three dimensional structures of λ Cro and the operator-binding domain of λ repressor has led to detailed molecular models for their complexes with operator (1-4). These models, which use the standard B-DNA geometry for operator DNA, neglect the possibility that the local structure and dynamics of the operator may play an important role in protein recognition. Indeed, sequence dependent local perturbations of the structure of DNA have recently been observed in the crystal state (17-20) and in solution (21-23). These local variations in the DNA geometry alter the spatial relationships among the functional groups available for protein binding.

The imino and adenine H2 resonances are sensitive to changes in the local

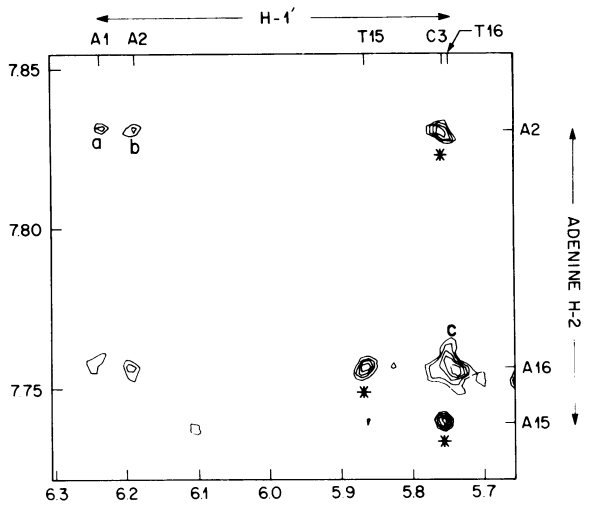

Figure 5. Cross-relaxation between adenine H2 and deoxyribose H1' protons is observed in this region of the 2D NOESY difference spectrum of O_L1 at 30°C. These effects are sensitive to distortions in local helical structure. Asterisks indicate NOEs between adenine H2 protons (A_2, A_{16}, and A_{15}) and H1' protons, assigned to their respective 3'-flanking sugars by the sequential method (6). Cross-peaks a and b are weaker effects between A_2H2 and the H1' protons of A_1 and its own sugar, respectively. Their relative intensities are consistent with distances inferred from single crystal studies. Cross-peak c contains unrelated effects between guanine H8 and H1' protons (6). The oligonucleotide was made 6 mM in Buffer A in D_2O solution. Mixing times of 300 and 5 ms were interweaved, and subtracted after appropriate scaling. The recycle delay was 1 second. A convolution difference was applied with gaussian broadening 2 hertz, exponential broadening 100 hertz, and substraction factor 0.8.

structure and dynamics of the double helix. The imino protons participate in Watson-Crick hydrogen bonding, and their resonances are shifted unusually far downfield by the diamagnetic ring currents of the stacked bases. Because of their position near the center of the DNA, their chemical shifts are sensitive to changes in relative base pair orientation. In addition, the lineshapes and relaxation behavior of these exchangable resonances are sensitive to the kinetics of base-pair fraying. It has recently been shown, moreover, that the NOE between the H2 protons of contiguous AT base pairs is a sensitive marker for base-pair propeller twisting (21). Similar distortions in local structural parameters significantly affect the distances between an H2 and its neighboring H1' protons (17-20). Thus, these NOE's may be useful in characterizing altered B-DNA structures in solution.

The assignments obtained here may be compared with those reported for an

Table 2. Summary of imino and adenine H2 assignments. Imino resonances α_n and γ_n are shown in Figure 1A. H2 assignments are for 30° C (Fig. 2). H2 chemical shifts vary with temperature in the pre-melting region (c.f. Fig. 4).

Base Pair	AH2 Assignment	Imino Assignment
5' T_1A_1 3'	7.605	α_{23}
A_2T_2	7.834	α_{78}
C_3G_3	--	γ_{67}
C_4G_4	--	γ_5
A_5T_5	7.579	α_4
C_6G_6	--	γ_8
T_7A_7	7.413	α_1
G_8C_8	--	γ_4
G_9C_9	--	γ_3
$C_{10}G_{10}$	--	γ_2
$G_{11}C_{11}$	--	γ_1
$G_{12}C_{12}$	--	γ_{67}
$T_{13}A_{13}$	7.547	α_5
$G_{14}C_{14}$	--	γ_9
$A_{15}T_{15}$	7.736	α_6
$T_{16}A_{16}$	7.755	α_{78}
3' $A_{17}T_{17}$ 5'	7.484	α_{23}

O_R3 operator site, which is in part homologous (13,14). These sequences may be aligned

O_L1 5' T A C C A C T G G C G G T G A T A 3'
O_R3 5' T A T C C C T T G C G G T G A T A 3'

The right halves of these two oligonucleotides are the same. Accordingly, some of their imino resonances are observed to be similar. Resonance γ_9 in the spectrum of O_L1, for example, is assigned to $G_{14}C_{14}$, as is the corresponding resonance in the spectrum of O_R3. These operator sites are recognized by λ repressor and Cro with opposite orders of affinity. Because these relative affinities play an important role in gene regulation, a comparison of operator structures would be of biological interest.

DNA protons constitute two cross-relaxation networks. The first and larger network consists of the major groove base protons and the sugar protons. We and others have proposed a sequential assignment strategy for

this network based upon two-dimensional methods (6,24-26). The second cross-relaxation network includes the imino and adenine H2 protons. The one-dimensional NOE methods developed by Redfield and coworkers (10,11) suffice to assign this network in small oligomers. However, for O_L1, an asymmetric 17mer, it is necessary to introduce two-dimensional techniques. The H1' deoxyribose protons, which lie at the edge of the minor groove, form a common relaxation pathway for both networks. This connection permits the sequential assignment of the major groove protons to be extended to the minor groove. Thus, two-dimensional NOE spectra in H_2O solution should in principle permit a single assignment strategy for all DNA protons, including the imino protons. Work to this end is in progress.

CONCLUSION

As a first step in the determination of the solution structure and dynamics of a synthetic λ operator site O_L1, an asymmetric duplex of 17 base pairs, the complete assignment of the imino and adenine H2 resonances is presented.

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